SUVmax or the T/M ratio because both SUVmax and the T/M ratio are calculated based on the maximum point SUV. The decrease in F-MISO SUVmax may be related to a decrease in blood flow following vascular damage due to RT. However, it has been demonstrated that the functional vascularity in human tumors remains unchanged or improves slightly during the early period of conventional fractionated RT with 1.5–2.0 Gy daily doses [20]. Therefore, we consider that the decrease in F-MISO accumulation indicates reoxygenation of tumor cells.

The information on tumor hypoxia from F-MISO PET/CT can be used for dose painting intensity modulated radiation therapy planning. Although some reports showed dose painting RT plans up to a maximum tumor dose of 84–105 Gy using F-MISO PET/CT [21, 22], no clinical trials for dose escalation to hypoxic areas have been reported. One reason for not performing such clinical trials is that the reproducibility of intratumor distribution of F-MISO is unclear [23]. Nehmeh *et al.* [24] found that only six of 13 human tumors showed well-correlated intratumor distributions of F-MISO after a three-day interval without treatment. The remaining seven tumors showed different distributions of F-MISO after three days. The other factor is reoxygenation of hypoxic areas during fractionated RT. Therefore, dose escalation to the hypoxic areas in the initial PET/CT before RT may be inappropriate. In the near future, if frequent imaging of F-MISO PET/CT is available, adaptive RT for tumor hypoxia may be used clinically.

F-MISO PET/CT obtained before or during fractionated RT can be used as an independent prognostic measure and has implications for treatment strategy. If tumor response can be predicted at the initial period of RT, the treatment strategy can be changed to a more intensive one or a substitute therapy can be started. In the present study, tumors with an SUVmax of >2.5 or an HV of > 6.0% in the second F-MISO study showed poor local response. Similar results were shown in various reports on head and neck cancer and lung cancer using pretreatment hypoxia imaging [25, 26]. The predictive value of hypoxic imaging should be evaluated in future studies.

In conclusion, accumulation of F-MISO of ≥1.60 SUV was regarded as an intratumoral hypoxic area in our F-MISO PET/CT system. Most human tumors (90%) in this small series had hypoxic areas before RT, although HV was minimal (0.0–0.3%) in four of the 10 tumors. In addition, reoxygenation was observed in most tumors at two weeks of fractionated RT.

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Cancer Medicine

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ORIGINAL RESEARCH

Human papillomavirus DNA and p16 expression in Japanese patients with oropharyngeal squamous cell carcinoma

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Keywords
DNA methylation, human papillomavirus, oropharynx, p16, squamous cell carcinoma

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Abstract

Human papillomavirus (HPV) is a major etiologic factor for oropharyngeal squamous cell carcinoma (OPSCC). However, little is known about HPV-related OPSCC in Japan. During the study, formalin-fixed, paraffin-embedded OPSCC specimens from Japanese patients were analyzed for HPV DNA by the polymerase chain reaction (PCR) and for the surrogate marker p16 by immunohistochemistry. For HPV DNA-positive, p16-negative specimens, the methylation status of the p16 gene promoter was examined by methylation-specific PCR. Overall survival was calculated in relation to HPV DNA and p16 status and was subjected to multivariate analysis. OPSCC cell lines were examined for sensitivity to radiation or cisplatin in vitro. The study results showed that tumor specimens from 40 (38%) of the 104 study patients contained HPV DNA, with such positivity being associated with tumors of the tonsils, lymph node metastasis, and nonsmoking. Overall survival was better for OPSCC patients with HPV DNA than for those without it (hazard ratio, 0.214; 95% confidence interval, 0.074–0.614; $P = 0.002$). Multivariate analysis revealed HPV DNA to be an independent prognostic factor for overall survival ($P = 0.015$). Expression of p16 was associated with HPV DNA positivity. However, 20% of HPV DNA-positive tumors were negative for p16, with most of these tumors manifesting DNA methylation at the p16 gene promoter. Radiation or cisplatin sensitivity did not differ between OPSCC cell lines positive or negative for HPV DNA. Thus, positivity for HPV DNA identifies a distinct clinical subset of OPSCC with a more favorable outcome in Japanese.

Introduction

Head and neck cancer is the sixth most common cancer worldwide, with an estimated annual incidence of approximately 600,000 cases [1]. Although the incidence of such cancer overall has fallen in recent years, consistent with the decrease in tobacco use, that of oropharyngeal squamous cell carcinoma (OPSCC) has increased in both the United States and Europe. In 2009, the International Agency for Research on Cancer recognized human papillomavirus (HPV) type 16 as a causal agent of OPSCC [2]. Individuals with HPV-positive OPSCC show significantly better overall survival and disease-free survival, associated with a 20–80% reduction in the risk of death, compared with those with HPV-negative OPSCC [3, 4]. Knowledge of HPV status in patients with OPSCC is thus expected to play an increasing role in the management of this disease. Epidemiological evidence from several countries indicates that the proportion of OPSCC cases caused by HPV varies widely, however. Although the proportion of

OPSCC cases attributable to HPV ranges from 40 to 80% in the United States and is around 90% in Sweden [3, 5], little is known about HPV-related OPSCC in Asian populations.

The aim of this study was to evaluate the prevalence, clinical features, and outcome of OPSCC positive for HPV DNA in the Japanese population. We also assessed the concordance between the presence of HPV DNA in tumor specimens and expression of the host cyclin-dependent kinase inhibitor p16 as detected by immunohistochemistry (IHC), given that p16 is commonly examined as a surrogate marker for HPV positivity in OPSCC [3, 6], and we further investigated possible mechanisms underlying any discordance. Moreover, to evaluate the biological impact of HPV infection, we examined the sensitivity of OPSCC cell lines positive or negative for HPV DNA to radiation and to cisplatin.

Material and Methods

Patients and tissue

With approval of the appropriate institutional review board, we analyzed formalin-fixed, paraffin-embedded (FFPE) tissue from 118 consecutive patients with newly diagnosed and histologically confirmed OPSCC who were treated at Kinki University Hospital from November 2000 through December 2011. Tumor specimens for all cases were obtained during surgery or diagnostic biopsy, and one representative paraffin block was selected for each case. Several 6-μm paraffin sections were used for analysis of HPV DNA, and one 3-μm section was used for p16 IHC. Patients without sufficient tumor tissue available for both analysis of HPV DNA and p16 staining were excluded, leaving 104 patients in the study (Fig. 1).

Clinicopathologic characteristics and outcome data for patients were obtained from the medical records. Treatment modality was selected for each patient individually on the basis of the official published guidelines. Most individuals underwent radiation therapy or radiochemotherapy according to a standard fractionated regimen, receiving 60–70 Gy with or without concomitant platinum-based chemotherapy. Adjuvant radiotherapy (54–64 Gy) was administered with standard fractionation.

Analysis of HPV DNA

The FFPE specimens were depleted of paraffin and then subjected to macrodissection in order to select a region of cancer tissue. Genomic DNA was extracted from the cancer tissue with the use of a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany), and the DNA concentration of each extract was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). DNA for HPV types 16, 18, 31, 33, and 35 was detected with the use of a TaqMan real-time polymerase chain reaction (PCR)-based method (Applied Biosystems, Foster City, CA) that was designed to amplify the E6 region or E7 region (or both) of the viral genome. The primer and probe sequences for amplification have been described previously [7–9]. Samples of genomic DNA that had sufficient amplifiable β-globin DNA (>1 human genome/μL) were considered to be evaluable, and HPV type was determined for β-globin gene-positive and HPV DNA-positive specimens. We defined active HPV DNA

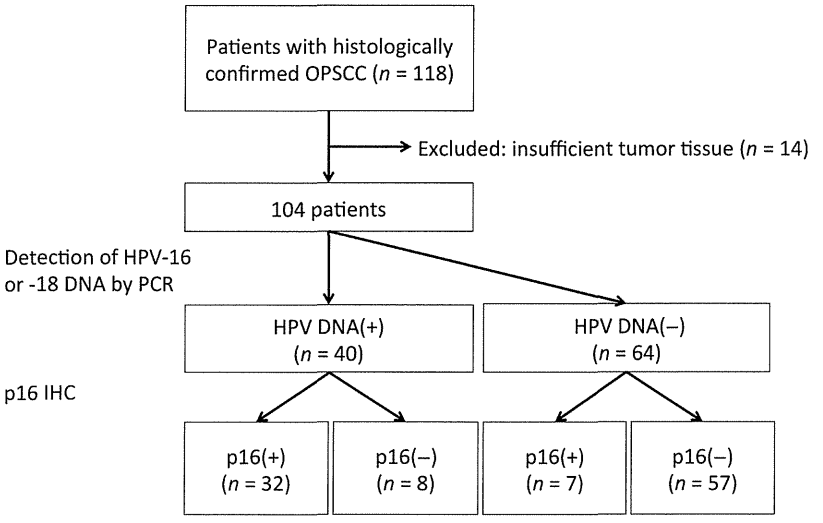

Figure 1. Summary of the protocol for classification of enrolled OPSCC patients according to HPV DNA and p16 status. OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus.

involvement as PCR detection at the level of at least one copy per 10 cell genomes [7]. PCR analysis was performed in duplicate.

IHC for detection of p16 expression

Immunohistochemistry for p16 was performed with the use of a CINtec Histology Kit (MTM Laboratories AG, Heidelberg, Germany) based on the monoclonal antibody E6H4. A tonsil squamous cell carcinoma with a high level of p16 expression was used as a positive control, and the primary antibody was omitted as a negative control. Expression of p16 was scored positive if strong and diffuse nuclear and cytoplasmic staining was present in >70% of the tumor cells [10], and p16 scoring was performed without knowledge of HPV status. Representative p16 IHC images are shown in Figure 2.

Methylation-specific-PCR analysis

For assessment of DNA methylation at the p16 gene promoter, genomic DNA samples were subjected to sodium bisulfite modification with the use of a MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures, Randwick, NSW, Australia). The modified DNA was then used as a template for methylation-specific (MS)-PCR with primers specific for methylated or unmethylated sequences [11]. The sizes of the MS-PCR products were previously described [12]. Real-time MS-PCR analysis was performed in a 25-μL reaction mixture with the use of an EpiScope MSP Kit (Clontech, Mountain View, CA). EpiScope Methylated HCT116 gDNA and EpiScope Unmethylated HCT116 DKO gDNA (Clontech) were used as positive and negative controls, respectively.

Cell culture

The human OPSCC cell lines UPCI-SCC-003, UPCI-SCC-036, UPCI-SCC-089, UPCI-SCC-090, UPCI-SCC-152, and UPCI-SCC-154 were kindly provided by S. Gollin (University of Pittsburgh School of Public Health, Pittsburgh, PA). The cells were cultured under an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 0.1 mmol/L nonessential amino acids (Gibco-Invitrogen, Carlsbad, CA), 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate (Sigma-Aldrich).

Clonogenic survival assay

Exponentially growing cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities, plated in triplicate in 25-cm^2 flasks containing 10 mL of complete medium, and exposed at room temperature to various doses of radiation with a ^{60}Co irradiator at a rate of ~0.82 Gy/min. The cells were cultured for 14–21 days, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted. The surviving fraction was calculated as: (mean number of colonies)/(number of plated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of plated cells for corresponding nonirradiated cells.

Cell growth inhibition assay

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of cisplatin for 72 h. Cell Counting Kit-8 solution

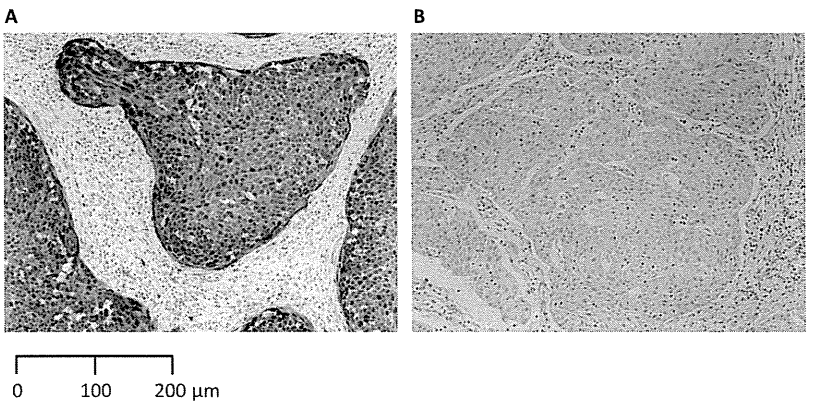

Figure 2. Representative IHC staining of p16 in OPSCC tumor specimens. Tumors were classified as either positive (A) or negative (B) for p16 expression. Scale bar, 200 μm. IHC, immunohistochemistry; OPSCC, oropharyngeal squamous cell carcinoma.

(Dojindo, Kumamoto, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA). Absorbance values were expressed as a percentage of that for nontreated cells, and the median inhibitory concentration (IC_{50}) of cisplatin for inhibition of cell growth was determined.

Statistical analysis

Patient characteristics were compared between individuals positive or negative for HPV DNA with Student's two-tailed *t*-test or the chi-square test. Survival curves were constructed by the Kaplan–Meier method and were compared with the log-rank test. The impact of various factors on survival was evaluated by multivariate analysis according to the Cox regression model. Concordance between HPV DNA and p16 assay results was assessed with the kappa statistic (κ) and Spearman correlation. Statistical analysis was performed with the use of IBM SPSS statistics software version 20 (SPSS Inc., IBM, Chicago, IL). A *P*-value of <0.05 was considered statistically significant.

Results

Patient characteristics

The characteristics of the 104 studied patients are listed in Table 1. The median age of the patients was 64 years,

Table 1. Characteristics of the 104 study patients according to HPV DNA and p16 status.

	All patients (n = 104), n (%)	HPV DNA(+) (n = 40, 38%), n (%)		HPV DNA(−) (n = 64, 62%), n (%)		HPV DNA(+) vs. HPV DNA(−) (*P*-value)
		p16(+) (n = 32)	p16(−) (n = 8)	p16(+) (n = 7)	p16(−) (n = 57)	
Sex						
Male	81 (78)	23 (72)	6 (75)	5 (71)	47 (82)	0.329
Female	23 (22)	9 (28)	2 (25)	2 (29)	10 (18)	
Age (years)						
Median	64	60	66	71	65	0.276
Range	35–80	36–75	35–71	59–77	38–80	
T classification						
1–2	70 (67)	23 (72)	6 (75)	6 (86)	35 (61)	0.372
3–4	34 (33)	9 (28)	2 (25)	1 (14)	22 (39)	
N classification						
0	29 (28)	3 (9)	3 (38)	1 (14)	22 (39)	0.021
1–3	75 (72)	29 (91)	5 (63)	6 (86)	35 (61)	
Stage						
I–III	27 (26)	6 (19)	3 (38)	1 (14)	17 (30)	0.524
IV	77 (74)	26 (81)	5 (63)	6 (86)	40 (70)	
Tobacco usage						
Never smoker	39 (38)	21 (66)	1 (13)	5 (71)	14 (25)	0.010[1]
<40 pack-years	27 (26)	9 (28)	2 (25)	0 (0)	16 (28)	
>40 pack-years	38 (37)	2 (6)	5 (63)	2 (29)	27 (47)	
Tumor location						
Tonsil	60 (58)	26 (81)	5 (63)	5 (71)	24 (42)	0.002[2]
Posterior wall	6 (6)	0 (0)	0 (0)	0 (0)	6 (11)	
Lateral wall	4 (4)	2 (6)	0 (0)	1 (14)	1 (2)	
Base of tongue	13 (13)	4 (13)	0 (0)	1 (14)	8 (14)	
Anterior palatine arch	13 (13)	0 (0)	2 (25)	0 (0)	11 (19)	
Unknown	8 (8)	0 (0)	1 (13)	0 (0)	7 (12)	
Initial therapy[3]						
RT(+)	85 (82)	27 (84)	8 (100)	7 (100)	43 (75)	0.426
RT(−)	19 (18)	5 (16)	0 (0)	0 (0)	14 (25)	

P-values were calculated with Student's two-tailed *t*-test for age and the chi-square test for other variables. HPV, human papillomavirus.
[1]Comparison of patients who never smoked versus patients with a smoking history.
[2]Comparison between tonsil and other sites.
[3]RT(+), treatment with radiation, including radiation therapy alone (n = 3), chemoradiotherapy alone (n = 13), or surgery followed by radiation therapy (n = 46) or by chemoradiotherapy (n = 23); RT(−), treatment without radiation, including surgery alone (n = 11), surgery followed by chemotherapy (n = 1), chemotherapy alone (n = 4), and best supportive care (n = 3).

with a range from 35 to 80 years, and most of them were male patients (78%) and had stage IV disease (74%).

Presence of HPV DNA and p16 expression in OPSCC

Of the 104 tumor specimens, 40 (38%) were positive for HPV-16 or HPV-18 DNA by PCR analysis (Fig. 1). These 40 tumors included 37 positive for HPV-16 alone, two positive for HPV-18 alone, and one positive for both HPV-16 and HPV-18. HPV DNA was detected more frequently in the tonsils ($P = 0.002$) than in other regions (Table 1). Patients positive for HPV DNA presented significantly more often with lymph node metastasis (85 vs. 64%, $P = 0.021$) and included a higher proportion of never-smokers (55 vs. 30%, $P = 0.010$) compared with those negative for HPV. There was no significant association between HPV DNA status and gender, age, T classification, or disease stage.

Expression of p16 was detected by IHC in a total of 39 tumors (Fig. 2). Of the 40 cases positive for HPV DNA, 32 (80%) were positive for p16, whereas 57 (89%) of the 64 cases negative for HPV DNA were also negative for p16 (Fig. 1). There was thus good agreement between HPV DNA positivity and p16 positivity ($\kappa = 0.65$; 95% confidence interval [CI], from 0.50 to 0.80; $r = 0.631$; $P < 0.001$).

DNA methylation at the p16 gene promoter in OPSCC

To identify the underlying mechanism of p16 gene silencing in tumors positive for HPV DNA but negative for p16 expression, we examined the DNA methylation status of the p16 gene promoter region with the use of MS-PCR analysis. Among the eight such cases, DNA methylation at the p16 gene promoter was detected in six (cases 66, 69, 71, 82, 96, and 106) (Fig. 3).

Survival analysis

Oropharyngeal squamous cell carcinoma patients positive for HPV DNA showed a significantly better overall survival compared with those negative for HPV DNA [hazard ratio (HR), 0.214; 95% CI, from 0.074 to 0.614; $P = 0.002$] (Fig. 4A). For OPSCC of stages I to III, HPV-positive patients tended to have a better overall survival compared with their HPV-negative counterparts, but the difference was not statistically significant ($P = 0.129$), possibly because of the small sample size ($n = 27$) (Fig. S1A). On the other hand, for OPSCC of stage IV

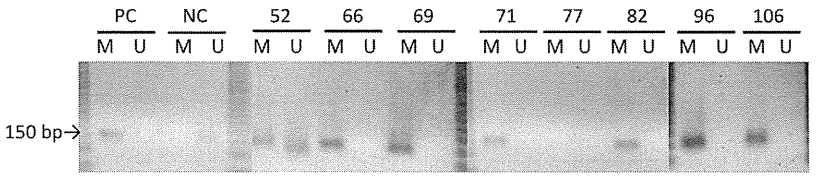

Figure 3. MS-PCR analysis of the p16 gene promoter in eight OPSCC tumors positive for HPV DNA but negative for p16 by IHC. The position of a 150-bp amplification product corresponding to the methylated promoter is indicated. PC, positive control; NC, negative control; M, methylated; U, unmethylated; MS-PCR, methylation-specific polymerase chain reaction; OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus; IHC, immunohistochemistry.

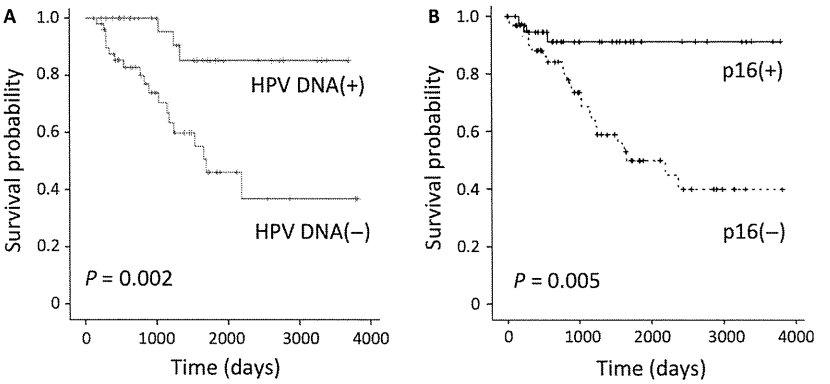

Figure 4. Kaplan–Meier curves for overall survival of OPSCC patients according to HPV DNA (A) or p16 (B) status. *P*-values were calculated by the log-rank test. OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus.

(n = 77), patients with HPV DNA showed a significantly better overall survival than did those without it (P = 0.002) (Fig. S1B). Stratification based on p16 expression also revealed a significantly better outcome for OPSCC patients positive for p16 than for those negative for this marker (HR, 0.245; 95% CI, from 0.085 to 0.705; P = 0.005) (Fig. 4B). To rule out potential confounding effects for the presence of HPV DNA and other factors, we performed multivariate analysis for overall survival (Table 2). The presence of HPV DNA was revealed to be an independent and significant prognostic factor for overall survival (HR, 0.248; 95% CI, from 0.080 to 0.766; P = 0.015) after taking into account gender, age, T and N classification, smoking history, tumor location, and radiation therapy.

Sensitivity of OPSCC cell lines with or without HPV DNA to radiation and cisplatin

We next investigated the biological impact of HPV DNA status with OPSCC cell lines positive (UPCI-SCC-090, -152, and -154) or negative (UPCI-SCC-003, -036, and -089) for HPV DNA. A clonogenic survival assay performed after exposure of the cells to various doses of radiation revealed no significant difference in survival between the cell lines positive or negative for HPV DNA (Fig. 5A). We also examined the effect of cisplatin on the growth of the cell lines, again detecting no difference in the IC_{50} value of cisplatin between those positive or negative for HPV DNA (Fig. 5B, Table 3).

Table 2. Multivariate analysis of overall survival in patients with OPSCC (n = 104).

	Overall survival		
Factor	HR	95% CI	P
HPV DNA (positive vs. negative)	0.248	0.080–0.766	0.015
Gender (female vs. male)	0.870	0.231–2.151	0.539
Age (≤63 vs. >64 years)	0.833	0.392–1.770	0.634
T classification (1–2 vs. 3–4)	0.718	0.315–1.640	0.432
N classification (0 vs. 1–3)	1.536	0.640–3.680	0.337
Smoking history (nonsmoker vs. smoker)	0.541	0.120–2.445	0.424
Tumor location (tonsil vs. other)	0.597	0.277–1.289	0.189
RT[1], RT(+) vs. RT(−)	2.233	0.390–13.89	0.355

OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus; HR, hazard ratio; CI, confidence interval.

[1]RT(+), treatment with radiation, including radiation therapy alone (n = 3), chemoradiotherapy alone (n = 13), or surgery followed by radiation therapy (n = 46) or by chemoradiotherapy (n = 23); RT(−), treatment without radiation, including surgery alone (n = 11), surgery followed by chemotherapy (n = 1), chemotherapy alone (n = 4), and best supportive care (n = 3).

Discussion

In this study, we applied PCR-based detection of viral DNA and IHC-based detection of p16 to tumor specimens from Japanese patients with OPSCC, given that this combination of approaches is the most reliable means to determine HPV status, with a sensitivity of 97% and specificity of 94% [13]. We found that 38% of the patients were positive for HPV DNA, consistent with recent studies that detected HPV DNA in 30–50% of OPSCC patients in Asian countries [14–16]. In the United States, the incidence of HPV-positive OPSCC increased by 225% from the late 1980s to the early 2000s [17], with 40–80% of OPSCCs now being caused by HPV [3]. This increase is thought to have resulted from the decrease in tobacco use and increased oral HPV exposure due to changes in sexual behavior among recent birth cohorts [3, 4]. As in other Asian countries, the prevalence of smoking in Japan is much higher than that in the United States, especially among men (32 vs. 17%) [18]. The lower proportion of OPSCC cases associated with HPV in Asian countries compared with Western countries might therefore be attributable, at least in part, to the difference in tobacco exposure. Given that the proportion of active smokers has recently been decreasing each year in Japan, the proportion of OPSCCs related to HPV in the Japanese population is likely to increase.

We found that overall survival for Japanese OPSCC patients positive for HPV DNA was significantly better than that for those negative for HPV DNA. The presence of HPV DNA was associated mostly with tumors of the palatine tonsils, lymph node metastasis, and nonsmoking. HPV-positive OPSCC was more frequent in younger individuals than was HPV-negative OPSCC, but the difference was not significant, possibly due to the relatively small sample size. These results are consistent with those for OPSCC in the United States and Europe [3, 4], suggesting similarity in the features of HPV-associated OPSCC between Japan and Western countries.

The reason for the more favorable prognosis of HPV-associated OPSCC remains unclear, although it may be related to a younger age at onset, minimal exposure to established risk factors such as cigarette smoking, or a better response to therapy [3, 19]. Indeed, recent studies have provided evidence that HPV-positive OPSCC shows a better response to chemotherapy [20, 21] or to radiotherapy either alone [22, 23] or in combination with chemotherapy [20, 21, 24, 25]. Although these findings are suggestive of an inherent radio- or chemosensitivity of HPV-positive OPSCC, we did not detect a difference in sensitivity to radiation or cisplatin in vitro between OPSCC cell lines positive or negative for HPV DNA. This apparent discrepancy between the in vitro and clinical

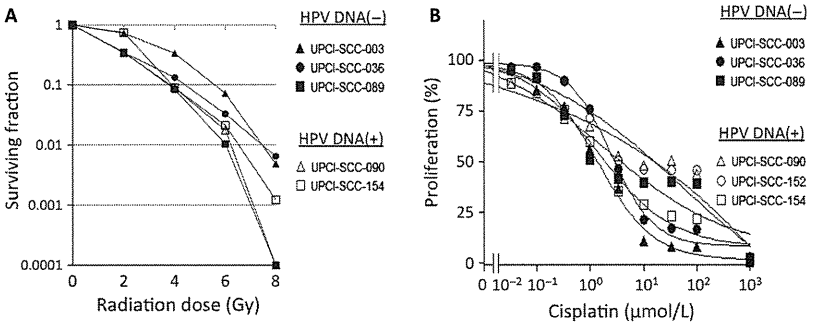

Figure 5. Sensitivity of OPSCC cell lines to radiation or cisplatin according to HPV DNA status. (A) Clonogenic assay for cells exposed to the indicated doses of radiation. This assay was not performed with UPCI-SCC-152 cells. (B) Effect of cisplatin concentration on cell growth. All data are means from three independent experiments. OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus.

Table 3. IC_{50} values of cisplatin for inhibition of OPSCC cell growth in vitro.

Cell line	HPV DNA	Cisplatin IC_{50} (μmol/L)
UPCI-SCC-003	(−)	1.7
UPCI-SCC-036	(−)	3.0
UPCI-SCC-089	(−)	1.2
UPCI-SCC-090	(+)	5.7
UPCI-SCC-152	(+)	4.6
UPCI-SCC-154	(+)	2.0

IC, inhibitory concentration; OPSCC, oropharyngeal squamous cell carcinoma; HPV, human papillomavirus.

data might be due to the limitations of in vitro assays, which do not accurately reflect the tumor microenvironment in vivo. Further study is thus needed to determine the molecular mechanism underlying the favorable outcome of patients with HPV-positive OPSCC, with the prospect that such knowledge might inform the development of therapeutic approaches to improve the poor prognosis of those with HPV-negative OPSCC.

In HPV-positive OPSCC, production of the viral oncoprotein E7 results in inactivation of the retinoblastoma (RB) protein and consequent upregulation of p16 expression [3, 26–28]. IHC positivity for p16 is thus associated with HPV-positive OPSCC, being regarded as a surrogate marker for HPV infection in such tumors [3, 6]. We also found a significant correlation between positivity for HPV DNA and IHC-based detection of p16 in Japanese patients with OPSCC, and the results of survival analysis based on p16 status as a stratification factor were similar to those of such analysis based on HPV DNA status.

Although most HPV-associated OPSCC tumors express p16, we found that 20% of HPV DNA-positive tumors (eight cases) were negative for p16 by IHC. A similar level of discordance was observed in previous studies based on the same approaches for detection of HPV DNA and p16 [7, 13, 29], although the underlying mechanism remains largely unknown. Given that DNA methylation at the p16 gene promoter has been identified as a key mechanism of p16 gene silencing in various types of primary tumor [30], we analyzed the methylation status of the p16 gene promoter in the eight tumors positive for HPV DNA but negative for p16 in this study with the use of MS-PCR analysis. We found a high frequency (6/8, 75%) of DNA methylation at the p16 gene promoter in these cases. As far as we are aware, this is the first demonstration of DNA methylation at the p16 gene promoter in OPSCC tumors positive for HPV DNA but negative for p16 by IHC. A recent meta-analysis showed that heavy cigarette consumption was associated with p16 gene methylation in patients with non-small cell lung cancer [12]. In this study, among the HPV DNA-positive subgroup, patients with tumors negative for p16 expression had a significantly more extensive smoking history than those with tumors positive for p16 ($P < 0.001$, Student's two-tailed t-test), suggesting that heavy smoking might be responsible, at least in part, for DNA methylation at the p16 gene promoter and a consequent loss of p16 expression. Consistent with the results of a previous study [7], we also found that the survival of patients with HPV DNA-positive, p16-negative tumors was not as good as that of those with HPV DNA-positive, p16-positive tumors (data not shown). These data thus suggest that IHC-based detection of p16 provides suboptimal prognostic information unless combined with PCR-based detection of HPV DNA.

Seven (11%) of the 64 HPV DNA-negative tumors in this study were positive for p16 by IHC. Given that the HPV DNA analysis was initially restricted to HPV types 16 and 18, we further investigated the possible presence of DNA for other high-risk types of HPV (types 31, 33, and 35), which, together with types 16 and 18, account for most cases of HPV-associated OPSCC [8, 13, 31]. However, none of the seven HPV DNA-negative,

p16-positive tumors was found to be positive for these other high-risk types of HPV (data not shown). Similar results have been obtained in previous studies based on detection of HPV by PCR or in situ hybridization [19], with a discordance rate of ~10–20%. Expression of p16 in such HPV DNA-negative tumors might reflect disturbances of the RB signaling pathway unrelated to HPV infection, as has been found to be the case in malignant lymphoma and small cell lung cancer [32]. The mechanism of p16 expression in the absence of detectable HPV DNA in OPSCC warrants further investigation.

Two prophylactic HPV vaccines against HPV types 6, 11, 16, and 18 (quadrivalent) or HPV types 16 and 18 (bivalent) have shown clinical efficacy for prevention of HPV-related cervical cancer [33] and anal cancer [34]. Both vaccines thus target HPV type 16, which accounts for >90% of HPV-associated OPSCCs [4]. Given the causal relation between HPV infection and OPSCC, clinical evaluation of the potential efficacy of HPV vaccines for reducing the incidence of HPV-associated OPSCC is warranted.

In conclusion, we found that 38% of Japanese patients with OPSCC are positive for HPV DNA, with such positivity being an independent prognostic factor for overall survival. Given that expression of p16 can be affected by genetic or epigenetic changes in addition to HPV infection, our results suggest that IHC-based detection of p16 provides suboptimal prognostic information if not combined with detection of HPV DNA. Further clinical studies are warranted to characterize the mechanism underlying the survival benefit conferred by HPV positivity in patients with OPSCC as well as to identify optimal treatments for this patient population.

Acknowledgment

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Kaplan–Meier curves for overall survival of patients with OPSCC of stages I to III (A) or of stage IV (B) according to HPV DNA status. *P*-values were calculated by the log-rank test.

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Alternating chemoradiotherapy in patients with nasopharyngeal cancer: prognostic factors and proposal for individualization of therapy

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The purpose of this study is to assess the efficacy of alternating chemoradiation in patients with nasopharyngeal cancer. From 1990–2006, 100 patients with nasopharyngeal cancer were treated with alternating chemoradiation at the Aichi Cancer Center. Of these, 4, 2, 23, 34, 13 and 23 patients were staged as I, IIA, IIB, III, IVA and IVB, respectively. The median radiation doses for primary tumors and metastatic lymph nodes were 66.6 Gy (range, 50.4–80.2 Gy) and 66 Gy (range, 40.4–82.2 Gy), respectively. A total of 82 patients received chemotherapy with both cisplatin and 5-fluorouracil (5-FU), while 14 patients received nedaplatin (CDGP) and 5-FU. With a median follow-up of 65.9 months, the 5-year rates of overall survival (OAS) and progression-free survival (PFS) were 78.1% and 68.3%, respectively. On multivariate analysis (MVA), elderly age, N3, and WHO type I histology proved to be significantly unfavorable prognostic factors of OAS. As for PFS, there were T4, N3, and WHO type I histology in MVA. Acute toxicities of hematologic and mucositis/dermatitis ≥ Grade 3 were relatively high (32%); however, they were well-managed. Late toxicities of ≥ Grade 3 were three (3%) mandibular osteomyelitis and one (1%) lethal mucosal bleeding. Results for alternating chemoradiation for nasopharyngeal carcinoma are promising. In order to improve outcomes, usage of intensity-modulated radiation therapy and application of active anticancer agents are hopeful treatments, especially for groups with poor prognosis factors with WHO type I histopathology, T4 and/or N3 disease.

Keywords: nasopharyngeal carcinoma; alternating chemoradiation; WHO type I histopathology

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a common disease among Southern Chinese, Southeast Asian, Northern African and Inuit populations. In Japan, the USA and Western European countries it is relatively rare. Because of anatomical characteristics, surgical treatment is very difficult. In addition, the majority of NPC patients revealed undifferentiated carcinoma, which is relatively sensitive to radiation therapy. Therefore, radiotherapy is widely accepted as the first choice of therapy for NPC. In recent years, by randomized-control trials, chemoradiotherapy has shown significant survival benefits over radiotherapy alone, improving both local and distant control [1–4]. In addition, meta-analysis of eight randomized trials showed significant benefits for OAS and event-free survival [5]. The pooled hazard ratio of death was 0.82 (95% confidence interval, 0.71–0.94; $P = 0.006$), corresponding to an absolute survival benefit of 6% at 5 y from the addition of chemotherapy. Thus, the standard treatment for locally advanced NPC is now believed to be concurrent chemoradiotherapy. However, several key factors need further clarification. Firstly, the chemotherapy used in the Intergroup 0099 study (IGS) consisted of three courses each of concurrent administration of cisplatin (CDDP) and adjuvant chemotherapy with both CDDP and 5-fluorouracil (5-FU). However, about two thirds (63%) of patients could receive concurrent chemotherapy, and about half (55%) could receive the full course of adjuvant chemotherapy. Secondly, a higher incidence of adverse events ≥ Grade 3 was observed in the chemoradiation group than in the radiation alone group (59% vs 34%). Finally, chemoradiation reduced distant metastasis; however, it did not reach sufficient levels. Of the 18 patients with recurrence in the

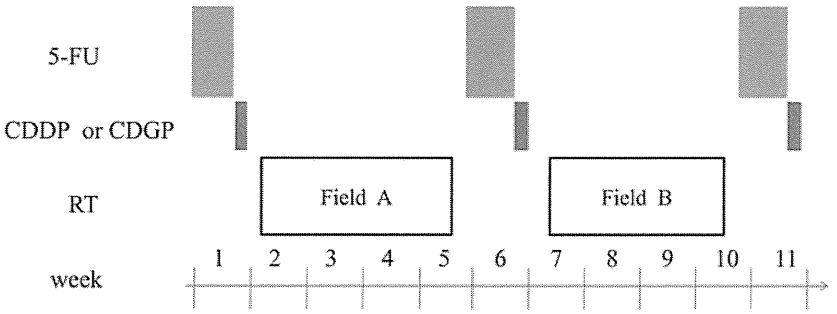

Fig. 1. Study design of alternating chemoradiotherapy. 5-FU = 5-fluorouracil 800 mg/m^2 on Days 1–5 continuous infusion, CDDP = cisplatin 50 mg/m^2 Day 6–7, CDGP = nedapatin 130 mg/m^2 on Day 6, RT = radiotherapy, Field A = large field including from the skull base to supraclavicular fossa, Field B = boost field including the nasopharynx and metastatic lymph nodes.

chemoradiation arm, 10 (56%) developed distant metastasis (DM) in the IGS. A considerable incidence of DM still developed in the IGS due to insufficient dose intensities of chemotherapy, instead of increasing adverse events.

In the Aichi Cancer Center, we conducted alternating chemoradiotherapy for advanced NPC patients from 1987 and reported promising results with sufficiently better compliance (94%), of which the 5-year OAS and PFS rates were 75% and 63%, respectively [6]. In the present study, we analysed the efficacy of alternating chemoradiotherapy for NPC with relatively longer follow-up and sought to refine our treatment strategy according to data regarding failure patterns.

MATERIALS AND METHODS

Patient characteristics

Between 1990 and 2006, a total of 100 consecutive patients with newly diagnosed histology-proven nasopharyngeal carcinoma underwent definitive chemoradiotherapy (CRT) in the Aichi Cancer Center. All patients underwent fiberoptic nasopharyngoscopy and magnetic resonance imaging (MRI) to assess the extent of primary and cervical lymph nodes. Evaluation of distant metastasis was done by chest X-ray, computed tomography (CT), liver ultrasonography, and bone scintigraphy. After 2002, positron emission tomography (PET) or PET-CT was also used to evaluate the extent of the disease. In addition, laboratory data, electrocardiograms, and 24-h creatinine clearance were evaluated to assess general condition. For this analysis, all patients were restaged according to the 6th edition of the American Joint Committee on Cancer (AJCC) staging system [6].

Treatment schedule

Chemotherapy

The treatment scheme is shown in Fig. 1. Details of the treatment regimen have been reported in another article [7]. Chemotherapy regimens were a combination of CDDP and 5-FU (FP) or nedaplatin (CDGP) and 5-FU (FN) regimens. In the FP regimen, 5-FU was administered continuously at a dose of 800 mg/m^2 on Days 1–5 and CDDP at a dose of 50 mg/m^2 on Days 6–7. In the FN regimen, 5-FU was administered continuously at a dose of 800 mg/m^2 on Days 1–5 and CDGP at a dose of 130 mg/m^2 on Day 6. Chemotherapy was performed in principal three times at 4-week intervals. However, when a WBC count <3000/mm^2 or a platelet count <100 000/mm^2 was obtained at the scheduled date of drug administration, chemotherapy was postponed and radiation therapy was alternately prescribed. When hematological data obtained two weeks after radiotherapy did not meet the inclusion criteria (WBC count >3000/mm^2 and platelet count >100 000/mm^2), the next cycle of chemotherapy was withdrawn. When the WBC count decreased to <1000/mm^2 or the platelet count decreased to <25 000/mm^2 after chemotherapy, doses of both 5-FU and CDDP were decreased by 25% at the next cycle. In addition, the dose of CDDP only was decreased by 25% when serum creatinine levels >1.5 mg/dl were noted.

Radiotherapy

Using a 6–10 MV photon beam by linear accelerator, external beam radiotherapy commenced 2–3 d after the completion of previous chemotherapy. At simulation and daily treatment, the head, neck and shoulder were immobilized in a hyperextended position using a thermoplastic mask. Radiotherapy was performed with a daily fraction of 1.8–2.0 Gy. The initial radiation field covered the nasopharynx and upper and middle cervical regions using bilateral opposing portals and lower cervical, and supraclavicular region using anterior single field irradiation at a dose of 36–40 Gy. Then, a shrinking field of 26–30 Gy was boosted to the nasopharynx and involved lymph nodes using the dynamic conformal rotational technique. In the shrinking field, we kept enough margins of primary tumors and involved lymph nodes from the edge of field. Those margins were mainly decided dependent on proximity to

critical structures such as the brain-stem, spinal cord, optic pathway and temporal lobes. During the second period of chemotherapy, radiotherapy was temporarily interrupted to spare the increasingly acute toxicity of 5-FU. Additional boosts of up to 10 Gy with stereotactic multiple arc treatment were also permitted, if residual tumors existed at primary sites.

Follow-up and statistical consideration

Toxicities of CRT were evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 [8]. During the treatment period, complete blood counts and biochemical examinations were performed at least once a week. After completion of CRT, the treatment response was assessed by fiberoptic nasopharyngoscopy, MRI and/or PET/CT. The frequency of follow-up was every month for the first year, once every two months between the second and third post-treatment year, and once every three months after the third post-treatment year. Fiberoptic nasopharyngoscopy was performed at every visit, and post-treatment MRI scans were obtained every three months for the first year and then every six months thereafter. The survival period was calculated from the start of treatment to death or the last follow-up examination, and progression-free survival was defined as the period from the start of treatment to the progression of tumors or death by any cause. Overall survival and progression-free survival curves were calculated by the Kaplan-Meier method [9]. The log-rank test was used to compare survival curves. A Cox-proportional hazard model was used for multivariate analysis. Differences in the ratios between the two groups were assessed by the chi-square test.

RESULTS

Patient characteristics

Between June 1990 and March 2005, 100 patients with NPC received definitive CRT in the Aichi Cancer Center. Table 1 shows patient characteristics in this cohort. We analysed all patients who were treated with CRT. The median age was 55 years old (range, 28–80). Performance status was distributed as 2 of 0, 93 of 1, 3 of 2, and 2 of 3, respectively. Of these, 8 patients (8%) had histopathology with keratinizing squamous cell carcinoma (WHO type I), and 70 patients (70%) had Stage III–IVB disease. During this period the number of patients with NPC who were treated with radiotherapy alone was 13. The common reasons for radiotherapy alone were advanced age or poor general condition.

Table 1. Patient characteristics

Characteristics		*n*
Age, years: median (range)	55 (28–80)	
Gender:		
Male		72
Female		28
Performance status		
0		2
1		93
2		3
3		2
Histology		
type I		8
non type I		90
others		2
T stage		
1		37
2a		15
2b		15
3		15
4		18
N stage		
0		11
1		31
2		34
3a		9
3b		15
Stage		
I		4
IIA		2
IIB		24
III		34
IVA		12
IVB		24

Treatment contents

The median dose to the primary site was 66.6 Gy (range, 50.4–80.2 Gy), and the median dose to involved lymph nodes was 66 Gy (range, 40.4–82.2 Gy), respectively. The median period of the whole course of alternating CRT was

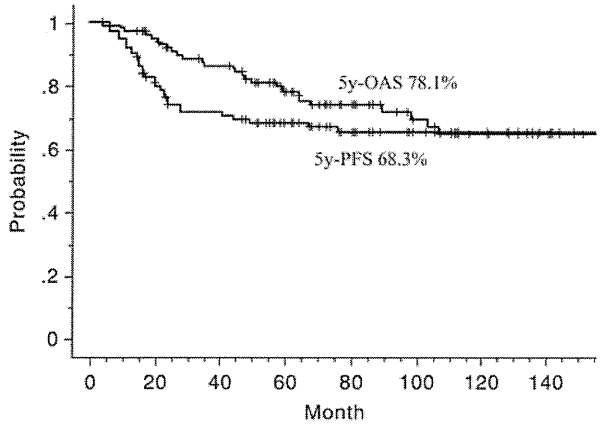

Fig. 2. Overall survival (OAS) and progression-free survival (PFS) curves.

85 days (range, 47–147 days), and the median period of overall treatment time of radiation therapy (OTT) was 69 days (range, 42–110 days).

Treatment outcomes

The 5-year rates of OAS and PFS were 78.1% and 68.3%, respectively (Fig. 2). The 5-year rates of OAS of the group divided by stage were 100, 100, 86.1, 77.6, 91.7 and 60.3% for Stage I, IIA, IIB, III, IVA and IVB, respectively. The 5-year rates of OAS and PFS of 96 patients who received alternating CRT were 78.2% and 68%, respectively. As for initial response after completion of CRT, complete remission (CR) rates of primary and nodal lesions were 86% and 83%, respectively. At a median follow-up of 65.9 months (range, 3.9–22.9 months), 62 were alive without disease, 11 were alive with disease, 18 died from the disease, 2 died from other diseases (both esophagus carcinoma) and 7 died from unknown reasons.

The 5-year rates of loco-regional progression-free survival (LRPFS) and distant metastasis-free survival (DMFS) were 77.9% and 87.8%, respectively.

A total of 32 patients (32%) developed treatment failure at one or more sites. Disease progression developed in 19 for primary, 9 for regional and 11 for distant sites at the last follow-up. Among 11 patients with distant failure, the most frequent site was the lung in 8, followed by bone in 4 and the liver in 2.

Of 21 patients who developed locoregional recurrence, 13 were treated with additional chemoradiation. Of the remainder, 2 patients were re-treated with radiotherapy alone, and 4 with only chemotherapy. One patient received neck dissection for regional failure, and another did not receive any treatment because of the patient's refusal for treatment.

Out of 11 patients who developed distant metastasis, 9 were treated by chemotherapy, and 2 patients received palliative radiotherapy only.

Univariate analysis

Univariate analysis (UVA) results are listed in Table 2.

Elderly age, male, WHO type I histology, and N3 were revealed as significant unfavorable prognostic factors of OAS. The 5-year rate of OAS of the group with WHO type I histology was significantly lower than that with non-type I histology (33.3% vs 81.6%, $P < 0.0001$, Fig. 3). The group with N3 lesions had significantly worse 5-year OAS (60.3%) than that with N0–2 (84%; $P = 0.0017$). The 5-year rates of OAS of patients who received reduced dose and planned dose chemotherapy were 76.6% and 78.6%, respectively ($P = 0.75$).

As for PFS, significantly unfavorable factors were revealed as WHO type I histology, T4 and N3.

The 5-year PFS rate of the group with N3 was significantly lower than that with N0–2 (41.5% vs 76.5%, $P = 0.001$). The 5-year PFS rate of the group with T4 was significantly lower than that with T1–3 (54.5% vs 71.4%, $P = 0.014$). The 5-year rates of PFS of patients who received reduced dose and planned dose chemotherapy were 69.7% and 66.7%, respectively ($P = 0.59$).

The 5-year rate of LRPFS of the group with WHO type I histology was significantly lower than that with non-type I histology (21.4 % vs 84.5 %, $P < 0.0001$).

The 5-year rate of DMFS of patients with N3 was significantly lower than that with N0–2 (62.8% vs 95.1%, $P < 0.0001$). The 5-year LRPFS of patients with T4 was significantly lower than that with T1–3 (63.3% vs 81.1%, $P = 0.027$).

Multivariate analysis

Multivariate analysis (MVA) results are listed in Table 3. On MVA, significantly unfavorable prognostic factors of OAS were elderly age, WHO type I histology and N3, respectively. As for PFS, they were WHO type I histology, T4 and N3, respectively.

Treatment compliance

Regarding the contents of chemotherapy, 82 patients received FP, while 14 received FN. Four patients had other chemotherapy regimens, as described below. One patient with Stage I (cT1N0M0) received two courses of CDDP/5-FU followed by definitive radiotherapy. One patient received six courses of weekly docetaxel (TXT) because of elderly age and poor medical condition. One patient received chemotherapy with both CDGP and TXT because 5-FU was inappropriate due to a past history of myocardial infarction. One patient received concurrent administration with decreased doses of CDGP and 5-FU due to elderly age. Chemotherapy compliance is shown in Table 4. In 96 patients who received alternating CRT, over 90% of patients received three courses of chemotherapy and 70% of patients received the planned dose of three courses. In

Table 2. Univariate analyses for overall survival and progression-free survival

Factors	No.	5-year OAS (%)	*P*-value	5-year PFS (%)	*P*-value
Gender					
Female	28	88.7	0.017	77.9	0.15
Male	72	73.8		64.4	
Age (years)					
<51	48	93.4	0.0006	73.6	0.26
≥51	52	64.2		63.4	
PS					
0, 1	95	79.1	0.148	69.9	0.1
2, 3	5	60		30	
Histology					
WHO non type I	90	81.6	$P<0.0001$	72.1	$P<0.0001$
type I	8	33.3		14.3	
T stage					
T1–3	82	78.2	0.79	71.4	0.014
≥T4	18	77.4		54.5	
N stage					
N0–2	76	84	0.001	76.5	0.001
N3	24	60.3		41.5	
Total treatment duration (day)					
<85	48	69	0.0615	62.3	0.135
≥85	52	85.6		73.8	
OTT (day)					
<69	49	78.2	0.884	72.2	0.36
≥69	51	78.2		64.8	
Dose for primary site (Gy)					
<66	30	76.7	0.712	70	0.7
≥66	70	78.7		67.5	
Dose for metastatic LN (Gy)					
<66	35	77.5	0.683	71.8	0.78
≥66	54	74.8		65.1	

OAS = overall survival, PFS = progression-free survival, PS = performance status, WHO = World Health Organization, OTT = overall treatment time of radiotherapy, LN = lymph node.

detail, 29 patients received reduced dose chemotherapy while 67 patients received the planned dose of three courses. The most common reason for dose reductions was renal dysfunction (47%), followed by severe mucositis (20%). The median total dose of CDDP was 300 mg/m^2 (range, 150–340 mg/m^2), CDGP was 375 mg/m^2 (range, 80–400 mg/m^2), and for 5-FU was 12 000 mg/m^2 (range, 3050–12 000 mg/m^2). In the cohort of patients who received reduced dose chemotherapy, the median total doses of CDDP, CDGP and 5FU were 250 mg/m^2, 330 mg/m^2 and 9400mg/m^2, respectively. Unplanned interruption of RT was experienced in 14 patients (14%), and 2 out of 14 patients required a break in RT over seven days. Severe mucositis (36%) was the most common reason for interruption of RT, followed by infection of the hyperalimentation catheter (29%).

Treatment toxicity

Acute toxicities observed during treatment are listed in Table 5. The most common toxicity was leukopenia. Grade

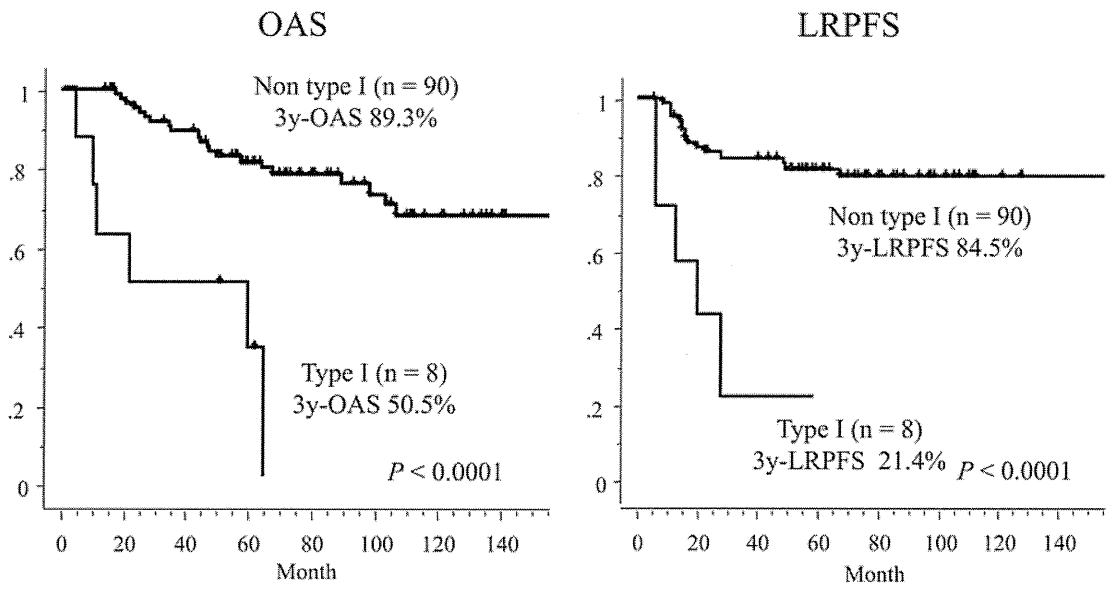

Fig. 3. Overall survival (OAS) and locoregional progression-free survival (LRPFS) curves of groups divided by WHO histopathological types.

Table 3. Multivariate analyses for overall survival and progression-free survival

		OAS		PFS	
Factors	**No.**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
Gender					
Female	28		0.109		0.5
Male	72	2.76 (0.104–1.257)		1.36 (0.291–1.836)	
Age (years)					
<51	48		0.0018		0.198
≥51	52	4.92 (0.074–0.551)		1.62 (0.294–1.290)	
Histology					
WHO non type I	90		0.0034		0.0004
type I	8	4.62 (0.077–0.603)		5.747 (0.067–0.454)	
T stage					
T1–3	82		0.555		0.023
T4	18	1.36 (0.264–2.047)		2.5 (0.181–0.881)	
N stage					
N0–2	76		0.0076		0.0025
N3	24	3.03 (0.147–0.745)		3.012 (0.163–0.680)	
OTT (day)					
<69	49	1.10 (0.395–2.065)	0.8092		0.605
≥69	51			1.215 (0.393–1.724)	

HR = hazard ratio, CI = confidence intervals, OAS = overall survival, PFS = progression-free survival, WHO = World Health Organization, OTT = overall treatment time of radiotherapy.

3 or higher leukopenia, neutropenia, thrombocytopenia and anemia occurred in 37, 22, 11 and 18 patients, respectively. Grade 3 or higher mucositis and dermatitis developed in 20 and 18 patients, respectively.

Late toxicities are listed in Table 6. Three Grade 3 osteomyelitis of the mandible occurred in this series. One patient died because of late toxicity due to lethal mucosal bleeding. The patient diagnosed as cT3N1M0 with histology of Type I received 80 Gy to the primary site including additional SRT boosts of 10 Gy due to an insufficient response at the planned 70 Gy. The patient developed active mucosal bleeding in the nasopharynx, and died five years later. We experienced no Grade 3 or higher late toxicity of brain necrosis, visual disturbance or swallowing disturbance.

DISCUSSION

A randomized control trial showed survival advantages of concurrent chemoradiotherapy over radiation alone, thus it is believed to be the standard treatment for locally advanced NPC. In the IGS, Stage III–IVB patients with NPC were randomized to CRT or RT, and the combined CRT group was treated with radiation and concurrent triweekly CDDP followed by three adjuvant cycles of FP [1]. The 3-year rate of OAS of the RT-only group was significantly lower than that of the CRT group (46% vs 76%; $P < 0.001$), and the same results were noted for the 3-year rate of PFS (24% vs 69%; $P < 0.001$). However, some problems with the results from the IGS were identified. Firstly, results of the RT arm in the IGS seem to be unacceptably bad because the reported 3-year rates of OAS for the same stages were over 70%. One of the reasons for this discrepancy is that the rate of WHO type I histology in the IGS series (24%) is larger than that of endemic regions, which is believed to have adversely impacted on clinical results. Secondly, the compliance of chemotherapy was insufficient in the IGS. The completion rates of planned chemotherapy of concurrent and adjuvant series were reported as 63% and 55%, respectively. In order to confirm this result, the IGS should be extrapolated in endemic regions [4]. In Hong Kong, the NPC-9901 trial on patients with T1-4N2-3M0 disease was designed to confirm the therapeutic ratio achieved by the IGS regimen. Regarding the compliance of chemotherapy, 65% of patients completed all six cycles, and 79% had five cycles. The CRT arm achieved significantly higher failure-free survival (72% vs 62% at 3 years, $P = 0.027$), mostly as a result of improvements in locoregional control. However, DMFS did not improve significantly (76% vs 73%, $P = 0.47$) and OAS was identical (78% vs 78%, $P = 0.97$). In other RCTs reported by Lin and Chen, the CRT arm significantly improved PFS and OAS [2, 3].

There is also evidence by meta-analysis dealing with eight randomized trials of 1753 patients regarding locally advanced NPC. In this analysis, the pooled hazard ratio of death for adding chemotherapy was 0.82 (95% confidence interval, 0.71–0.94; $P = 0.006$), corresponding to an absolute survival benefit of 6% at 5 years (56% vs 62%). A

Table 4. Compliance of chemotherapy

	n	median (range)
Total cycles given		
1	2	
2	7	
≥3	87	
Total dose given		
Cisplatin (mg/m^2)		300 (150–340)
Nedaplatin (mg/m^2)		375 (80–400)
5-fluorouracil (mg/m^2)		12 000 (3050–12 000)

Table 5. Acute, severe and life-threatening toxicities due to chemoradiotherapy

Toxicity	Gr 0	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5	unknown	≥ Gr 3
Leukopenia	4	12	43	32	5	0	4	37
Granulocytopenia	18	27	28	17	5	0	5	22
Anemia	6	33	39	14	4	0	4	18
Thrombocytopenia	28	37	10	8	3	0	4	11
Liver dysfunction	71	20	5	1	0	0	1	1
Renal dysfunction	71	28	0	0	0	0	1	0
Vomiting	33	14	50	3	0	0	0	3
Mucositis	0	13	67	19	1	0	0	20
Dermatitis	0	37	45	17	1	0	0	18
Salivary gland changes	1	13	86	0	0	0	0	0

Table 6. Late, severe and life-threatening toxicities due to chemoradiotherapy

Toxicity	Gr 0	Gr 1	Gr 2	Gr 3	Gr 4	Gr 5	≥ Gr 3
Swallowing dysfunction	95	4	1	0	0	0	0
Visual dysfunction	99	0	1	0	0	0	0
Hearing impairment	81	5	14	0	0	0	0
Osteomyelitis	96	0	1	3	0	0	3
Brain necrosis	99	1	0	0	0	0	0
Bleeding	99	1	0	0	0	1	1

significant interaction was observed between the timing of chemotherapy and overall survival ($P = 0.005$), with the highest benefit resulting from concomitant chemotherapy [5]. However, increasing acute toxicities caused by administration of chemotherapy were also reported in this analysis. In the IGS, acute toxicities of ≥ Grade 3 were reported as 50% and 76% for RT and CRT arms, respectively. Similarly, in the NPC-9901 trial, toxicities of ≥ Grade 3 were observed as 53% and 84% for RT and CRT arms, respectively ($P < 0.01$). The 3-year actuarial rate of late toxicity was slightly higher in the CRT arm than in that of the RT arm, although it was not significant (28% vs 13%, $P = 0.24$).

In our institute, we adopted alternating CRT for NPC from 1987. In a previous report, 32 patients with NPC received alternating CRT, and the 5-year rates of OAS and PFS were 75% and 63%, respectively. A Phase II study of alternating chemoradiotherapy for patients with NPC was performed in four medical institutions including our institution from 1997 and reported promising results with high compliance (91%), of which the 2-year OAS and PFS rates were 94% and 83%, respectively [10]. In the present study with longer follow-up and a larger cohort, the 5-year rates of OAS and PFS were 78.1% and 68.3%, respectively. We think these data are comparable with previous series. In addition, we believe that acute and late complication rates were sufficiently low according to longer follow-up with 65.9 months.

We believe alternating chemoradiotherapy has several advantages in CRT for NPC. Because the radiation field has to be large, severe mucositis and dermatitis sometimes develops and leads to a treatment break. In addition, late complications, such as disturbances in swallowing or hearing sometimes become significant problems. Alternating chemoradiotherapy has the potential benefit in reducing acute toxicities. As for reported data of the NPC-9901 trial, acute mucositis and skin reactions over Grade 3 were observed in 62% and 20% patients in the CRT arm, respectively. In the present study, acute mucositis or dermatitis of ≥ Grade 3 developed in 20% and 18%, respectively. By alternating chemotherapy and radiotherapy, we could also use intensive multi-agent chemotherapy regimens such as FP or FN without increasing acute and late complications. Although our data is a retrospective analysis in a single institute, the 5-year rate of OAS in the present study (78.1%) was more promising than that of the IGS trial (67%). Regarding the compliance of chemotherapy, over 90% patients in the present study could receive three courses of chemotherapy and 70% of our cohort had completed planned full doses. As a result the total dose of chemotherapy in patients who received a reduced dose was still about 80% of the planned dose. Our data is thought to be more encouraging than that of the IGS, in which only 55% patients completed the planned chemotherapy. Failure patterns in CRT for NPC patients are thought to be both loco-regional, but also in distant sites. In the present study, DMFS at 5-years was 87.8%, which was higher than that of the reported series. The 3-year DMFS rate of the NPC-9901 study was reported as 76%. We believe that it was caused by the advantages of intensive chemotherapy in the present study. An unexpected RT break was needed in 14 patients (14%), of which only 2 patients needed RT breaks longer than one week.

The argument against alternating CRT is that planned RT interruptions may lead to sacrifices in treatment efficacy. In many studies, it is well known that prolongation of overall treatment time negatively influences clinical outcomes. *In vitro*, accelerated repopulation occurred 28 days after the start of RT; thus, prolongation of treatment time led to the development of radiation resistance. In the present study, OTT was not significantly related to clinical outcome. One of the reasons is that the high compliance of the present study would have helped avoid essential prolongation of OTT in our cohort.

In the present series, WHO type I histopathology was a significantly unfavorable factor of both OAS and PFS. The incidence of WHO type I histology in Western countries is very different from East Asian countries. In the IGS series conducted in North America, the rate of WHO type I histology was 22%, which was higher than the rates in studies

conducted in endemic regions. WHO type I histopathology, keratinizing squamous cell carcinoma, was reported to be much less related to EBV infection than non-keratinizing carcinoma. It was also reported to be less sensitive to RT [11]. However, there are not so many reports regarding clinical results. One of the reasons is that the proportion of type I histopathology is very low in endemic regions. In Japan, the proportion of type I histopathology is about 20%, which was similar to North America. Kawashima *et al.* reported a Japanese multi-institutional survey of 333 NPC patients, in which the proportion of type I histopathology was 19% [12]. In that series, type I histopathology proved to be a significantly worse prognostic factor of OAS and PFS on both UVA and MVA. In the present study, the population of type I histopathology was 8%; however, these eight patients had remarkably poor prognosis. Six of the eight patients developed treatment failure. In our series, WHO type I histopathology was a significantly worse factor of both OAS (3-year rates; 50.5% vs 89.3%; $P < 0.0001$) and LRPFS (3-year rates; 21.4% vs 84.5%, $P < 0.0001$). The majority of failure patterns of these patients were in loco-regional sites. In order to improve treatment outcomes of these patients, dose escalation without increasing adverse events is believed to be promising. In recent years, intensity-modulated radiation therapy (IMRT) is widely used for head and neck cancer because of its dose conformity ability for PTV, reducing doses to normal tissue. RTOG 0225, a multi-institutional Phase II trial was conducted to test the feasibility of IMRT with or without chemotherapy for NPC. A 90% LRPF rate was reported as well as an acceptably low incidence of Grade 3 adverse events without xerostomia of Grade 4 [13]. In our institution, we started IMRT for NPC patients using Helical Tomotherapy until June 2006, and we have reported our preliminary clinical results [14]. In the future, dose escalation for patients with type I histopathology using IMRT will be helpful for improving clinical results.

The 5-year rates of PFS and LRPFS of patients with T4 were significantly inferior to those with T1–3, even though there was no significant difference in the 5-year rates of DMFS between these two groups. Because of the proximity of tumors to critical structures such as the brain-stem, spinal cord, optic pathway and temporal lobes, the radiation fields and dose coverages for primary tumors are often compromised. Preliminary results of radiation dose escalation for patients with T3–T4 NPC show good local control (2-year rate of locoregional control; 95.7%) and survival (2-year rate of OAS; 92.1%) [15]. For these patients, dose escalation using IMRT is also promising improved clinical results.

The 5-year rates of OAS and DMFS of patients with N3 were significantly inferior to those with N0–2 in the present series. On the other hand, N3 showed no apparent correlation with worsening LRPF. From this result, patients with N3 are expected to have a higher incidence of distant metastasis. Thus, a more effective regimen of chemotherapy should be considered to overcome limitations. In fact, TAX 324, a randomized Phase III trial, has shown the distinct survival advantages of multi-agent intensive chemotherapy including docetaxel and FP over PF for locally advanced head and neck cancer [16].

We believe that the present results for alternating chemoradiotherapy are promising compared to previously reported series of concurrent chemoradiotherapy. However, several subgroups with some risk factors proved to have insufficient outcomes. In order to refine clinical results without increasing adverse events, there is room for modification especially in patients with high-risk factors. Dose escalation using IMRT for type I histopathology and/or T4 disease and more intensive modifications of chemotherapy for N3 disease should be considered in future.

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